

Application
for
United States Letters Patent

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To all whom it may concern:

*Be it known that Catherine Dulac and Richard Axel
have invented certain new and useful improvements in
CLONING OF VERTEBRATE PHEROMONE RECEPTORS AND USES THEREOF*

of which the following is a full, clear and exact description.

CLONING OF VERTEBRATE PHEROMONE RECEPTORS
AND USES THEREOF

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Sub A1

This application claims the benefit of U.S. Provisional Application No. 60/005,698, filed October 19, 1995, the content of which is incorporated into this application by reference.

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The invention disclosed herein was made with Government support under NIH Grant No. NS 29832-04 from the Department of Health and Human Services. Accordingly, the U.S. Government has certain rights in this invention.

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Background of the Invention

Throughout this application, various references are referred to by abbreviation. Disclosures of these publications in their entireties are hereby incorporated by references into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citation for these references may be found at the end of this application, preceding the claims.

In mammals, olfactory sensory perception is mediated by two anatomically and functionally distinct sensory organs: the main olfactory epithelium (MOE) and the vomeronasal organ (VNO). Pheromones activate the VNO and elicit a characteristic array of innate reproductive and social behaviors, along with dramatic neuroendocrine responses. Differential screening of cDNA libraries constructed from single sensory neurons from the rat VNO has led to the isolation of a family of about 30 putative receptor genes. Sequence analysis indicates that these genes comprise a novel family of seven transmembrane

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domain proteins unrelated to the receptors expressed in the MOE. Moreover, the expression of each member of the gene family is restricted to a small subpopulation of VNO neurons. These genes encode mammalian pheromone
5 receptors.

Sensory systems receive information from the environment and transmit these signals to higher cortical centers in the brain where they are processed to provide an internal
10 representation of the external world. Mammals possess an olfactory system of enormous discriminatory power. Humans, for example, are capable of recognizing thousands of discrete odors. The perception of odors in humans is often viewed as an aesthetic sense, a sense capable of evoking emotion and memory leading to measured thoughts
15 and behaviors. Smell, however, is also the primal sense. In most species, odors can elicit innate and stereotyped behaviors that are likely to result from the nonconscious perception of odors. These different pathways of
20 olfactory sensory processing are thought to be mediated by two anatomically and functionally distinct olfactory sensory organs, the main olfactory epithelium (MOE) and the vomeronasal organ (VNO) (Figure 1).

25 In mammals, the sensory epithelium of the main olfactory system resides within the posterior recess of the nasal cavity, whereas the vomeronasal organ resides more anteriorly in a blind-ended pouch within the septum of the nose (Jacobson, 1811; see also Halpern, 1987;
30 Wysocki, 1989; and Farbman, 1992 for reviews). The sensory neurons of both the MOE and VNO are bipolar. The dendrites terminate in specialized microvilli or cilia that bind odorants and transduce specific odorant binding into neural activity. The axons from sensory neurons of
35 the MOE project through the skull to the main olfactory

bulb, the first relay station in the brain. The main olfactory bulb then sends most of its fibers to the olfactory cortex which in turn projects to higher sensory centers. The vomeronasal system, however, transmits
5 olfactory information via a separate pathway of neuronal projections. The neurons of the VNO send axons to the accessory olfactory bulb which projects to a discrete locus within the amygdala distinct from the zone that receives fibers from the main olfactory pathway
10 (Broadwell, 1975; Scalia and Winans, 1975; Winans and Scalia, 1970). The vomeronasal nucleus in the amygdala, in turn, sends fibers directly to the hypothalamus (Kevetter and Winans, 1981; Krettek and Price, 1977; 1978). Thus, the VNO pathway bypasses higher cognitive
15 centers resulting in innate and stereotyped behavioral and neuroendocrine responses.

What chemical signals activate the VNO and what responses do they elicit? The VNO is largely responsive to
20 olfactory cues secreted by other individuals within a species. These chemical signals provide information about gender, dominance, or reproductive status and elicit innate social and sexual behaviors, along with profound neuroendocrine changes (reviewed in Halpern,
25 1987; Wysocki, 1989; Wysocki and Lepri, 1991). In male rodents, for example, removal of the vomeronasal organ in virgin animals severely impairs sexual responses resulting in a dramatic reduction in the frequency of mating (Clancy et al., 1984; Meredith, 1986). In female
30 rodents, activation of the VNO can induce puberty and estrus in the presence of males and prevent estrus in group-housed females (Lomas, 1982; Johns et al., 1978; Reynolds and Keverne, 1979). Similarly, lesions in the vomeronasal system dramatically diminish male-specific
35 aggressive behaviors (Bean, 1982; Clancy et al., 1984).

The chemical signals responsible for eliciting these behaviors have been broadly defined as pheromones. Two classes of steroids, 16-androstenes and estrogens can elicit reproductive behaviors in some mammals (Melrose et al., 1971; Michael and Keverne, 1968); F-prostaglandins and steroids elicit sperm production and mating in fish (Stacey and Sorensen, 1986; Sorensen et al., 1988) and small fatty acids in association with the protein, aphrodisin, have been implicated in the male sexual response in hamsters (Henzel, et al., 1988; Singer, 1991). In most instances however, the chemical nature of the odorants responsible for innate behavioral responses has not been elucidated.

Neither the pheromone receptors nor the signal transduction pathways activated by pheromone in vomeronasal neurons have been identified. In the MOE, the repertoire of odorant receptor genes consists of about 1,000 genes, each encoding a distinct seven transmembrane domain protein (Buck and Axel, 1991; Parmentier et al., 1992; Ben Arie et al., 1994). Analysis of the expression patterns of this family of odorant receptor genes (Ngai et al., 1993; Ressler et al., 1993; Vassar et al., 1993; Vassar et al., 1994; Ressler et al. 1994), coupled with earlier electrophysiologic and tracing experiments (Kauer et al., 1987; Stewart et al., 1979; Lancet et al., 1982; Mori et al., 1992; Imamura et al., 1992; Katoh et al., 1993) have provided a logic for olfactory discrimination. Individual sensory neurons in the MOE are likely to express only one of the thousand receptor genes (Ngai et al., 1993; Chess et al., 1994; C. Dulac and R. Axel, unpublished studies). Neurons expressing a given receptor, although randomly distributed in domains of the epithelium, project their axons to a small number of

topographically fixed loci (or glomeruli) in the main olfactory bulb (Vassar et al, 1994; Ressler et al., 1994). These data support a model of olfactory coding in which discrimination of odor quality would result from
5 the detection of specific spatial patterns of activity in the olfactory bulb.

The isolation of the genes encoding the pheromone receptors from VNO neurons might similarly provide
10 insight into the chemical nature of the pheromones themselves, the logic of olfactory coding in the VNO, and the way in which perception of this class of odors leads to innate behaviors. Applicants' efforts to identify the genes encoding the mammalian pheromone receptors by
15 virtue of potential homology with the family of odorant receptor genes expressed in the main olfactory epithelium have been unsuccessful. Applicants therefore developed a cloning strategy in which cDNA libraries were constructed from individual rat VNO neurons. Difference
20 cloning permitted the identification of about 30 genes that define a novel family of presumed seven-transmembrane domain receptors that are evolutionarily independent of the odorant receptors of the MOE. Expression of the individual members of this gene family
25 is restricted to a distinct set of VNO neurons such that different neurons express different receptor genes. These genes encode mammalian pheromone receptors.

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Summary of the Invention

- This invention provides an isolated nucleic acid molecule encoding a vertebrate pheromone receptor. This invention
5 also provides a nucleic acid molecule of at least 12 nucleotides capable of specifically hybridizing with a unique sequence within the sequence of the above-described nucleic acid molecule.
- 10 This invention provides a vector which comprises the above-described isolated nucleic acid molecule. In an embodiment, the above described isolated nucleic acid molecule is operatively linked to a regulatory element. In a further embodiment, the vector is a plasmid.
- 15 This invention provides a host vector system for the production of a polypeptide having the biological activity of a vertebrate pheromone receptor which comprises the above-described vector and a suitable host.
- 20 This invention also provides a host vector system, wherein the suitable host is a bacterial cell, yeast cell, insect cell, or animal cell.
- 25 This invention provides a method of producing a polypeptide having the biological activity of a vertebrate pheromone receptor which comprising growing the above-described host vector system under conditions permitting production of the polypeptide and recovering the polypeptide so produced.
- 30 This invention also provides a purified, vertebrate pheromone receptor.
- 35 This invention also provides a polypeptide encoded by the above-described isolated vertebrate nucleic acid

molecule.

This invention provides an antibody capable of binding to
a vertebrate pheromone receptor. This invention further
5 provides an antibody capable of competitively inhibiting
the binding of the antibody capable of binding to a
vertebrate pheromone receptor.

This invention provides a method for identifying cDNA
10 inserts encoding pheromone receptors comprising: (a)
generating a cDNA library which contains clones carrying
cDNA inserts from an individual vomeronasal sensory
neuron; (b) hybridizing the nucleic acid molecules of
clones from the cDNA libraries generated in step (a) with
15 probes prepared from the individual vomeronasal neuron
and probes from a second individual vomeronasal neuron or
a main olfactory epithelium neuron; (c) selecting clones
which hybridized with probes from the individual
vomeronasal neuron but not from the second individual
20 vomeronasal neuron or the main olfactory epithelium
neuron; and (d) isolating clones which carry the
hybridized inserts, thereby identifying the inserts
encoding pheromone receptors.

25 This invention also provides the above-described method
wherein after step (c), further comprising: (a)
amplifying the inserts from the selected clones by
polymerase chain reaction; (b) hybridizing the amplified
30 inserts with probes from the individual vomeronasal
neuron; and (c) isolating the clones which carry the
hybridized inserts, thereby identifying the inserts
encoding the pheromone receptors. This invention also
provides cDNA inserts identified by the above methods.

35 This invention also provides a method for identifying DNA

inserts encoding pheromone receptors comprising: (a) generating DNA libraries which contain clones carrying inserts from a sample containing vomeronasal sensory neuron(s); (b) contacting clones from the cDNA libraries generated in step (a) with nucleic acid molecule of at least 12 nucleotides capable of specifically hybridizing with a unique sequence within the sequence of a pheromone receptor in appropriate conditions permitting the hybridization of the nucleic acid molecules of the clones and the nucleic acid molecule; (c) selecting clones which hybridized with nucleic acid molecule; and (d) isolating the clones which carry the hybridized inserts, thereby identifying the inserts encoding the pheromone receptors.

In an embodiment, the sample only contain an individual vomeronasal sensory neuron.

This invention also provides a method to identify DNA inserts encoding pheromone receptors comprising: (a) generating DNA libraries which contain clones with inserts from a sample containing vomeronasal sensory neuron(s); (b) contacting the clones from the DNA libraries generated in step (a) with appropriate polymerase chain reaction primers capable of specifically binding to nucleic acid molecules encoding pheromone receptors in appropriate conditions permitting the amplification of the hybridized inserts by polymerase chain reaction; (c) selecting the amplified inserts; and (d) isolating the amplified inserts, thereby identifying the inserts encoding the pheromone receptors.

This invention also provides DNA inserts identified by the above methods.

This invention provides a method to isolate DNA molecules encoding pheromone receptors comprising: (a) contacting

a biological sample known to contain nucleic acids with appropriate polymerase chain reaction primers capable of specifically binding to nucleic acid molecules encoding pheromone receptors in appropriate conditions permitting

5 the amplification of the hybridized molecules by polymerase chain reaction; (b) isolating the amplified molecules, thereby identifying the DNA molecules encoding the pheromone receptors. This invention also provides the nucleic acid molecules isolated by the above method.

10 This invention provides a method of transforming cells which comprises transfecting a host cell with a suitable vector comprising a nucleic acid molecule encoding a pheromone receptor as described above. This invention
15 also provides transformed cells produced by above method.

The invention also provides transformed cells wherein the host cells are not usually expressing pheromone receptors and transformed cells wherein the host cells are
20 expressing pheromone receptors.

This invention provides a method of identifying a compound capable of specifically binding to a vertebrate pheromone receptor which comprises contacting a
25 transfected cells or membrane fractions of the above transfected cells with an appropriate amount of the compound under conditions permitting binding of the compound to such receptor, detecting the presence of any such compound specifically bound to the receptor, and
30 thereby determining whether the compound specifically binds to the receptor.

This invention provides a method of identifying a compound capable of specifically binding to a vertebrate pheromone receptor which comprises contacting an
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appropriate amount of the purified pheromone receptor with an appropriate amount of the compound under conditions permitting binding of the compound to such purified receptor, detecting the presence of any such compound specifically bound to the receptor, and thereby determining whether the compound specifically binds to the receptor.

This invention also provides a method of identifying a compound capable of activating the activity of a pheromone receptor which comprises contacting the transfected cells or membrane fractions of the above described transfected cells with the compound under conditions permitting the activation of a functional pheromone receptor response, the activation of the receptor indicating that the compound is capable of activating the activity of a pheromone receptor.

This invention provides a method of identifying a compound capable of activating the activity of a pheromone receptor which comprises contacting a purified pheromone receptor with the compound under conditions permitting the activation of a functional pheromone receptor response, the activation of the receptor indicating that the compound is capable of activating the activity of a pheromone receptor.

This invention provides a method of identifying a compound capable of inhibiting the activity of a pheromone receptor which comprises contacting the transfected cells or membrane fractions of the above described transfected cells with an appropriate amount of the compound under conditions permitting the inhibition of a functional pheromone receptor response, the inhibition of the receptor response indicating that the

compound is capable of inhibiting the activity of a pheromone receptor.

This invention provides a method of identifying a compound capable of inhibiting the activity of a pheromone receptor which comprises contacting an appropriate amount of the purified pheromone receptor with an appropriated amount of the compound under conditions permitting the inhibition of a functional pheromone receptor response, the inhibition of the receptor response indicating that the compound is capable of activating the activity of a pheromone receptor. In an embodiment of the above method, the purified receptor is embedded in a lipid bilayer.

15 This invention also provides compounds identified by the above methods. This invention further provides a pharmaceutical composition comprising an effective amount of the identified compound and a pharmaceutically acceptable carrier.

20 This invention provides a method for manipulating the maternal behavior of a female subject comprising administering effective amount of the above compound to the female subject. In an embodiment, the female subject is a human.

25 This invention provides a method for manipulating the social behavior of a subject comprising administering effective amount of the above compound to the subject. In an embodiment, the subject is a human. In another embodiment, the subject is an animal.

30 This invention provides a method for manipulating the reproductive functions of an animal comprising

administering effective amount of the above compound to the subject. This invention provides a method for manipulating the reproductive behaviors of a subject comprising administering effective amount of the above compound to the subject.

This invention provides a method for increasing the fertility of a subject comprising administering effective amount of the above compound to the subject. This invention provides a method for manipulating hormonal secretion of a subject comprising administering effective amount of the above compound to the subject.

This invention also provides a method for manipulating food intake rate of a subject comprising administering effective amount of the above compound to the subject.

The above methods of different uses of the identified compounds are applicable to different domestic animals as well as human.

This invention provides a composition for manipulating the maternal behavior of a female subject comprising effective amount of the identified compound and an acceptable carrier. This invention also provides a composition for manipulating the social behavior of a subject comprising effective amount of the identified compound and an acceptable carrier. This invention further provides a composition for manipulating the reproductive functions of a subject comprising effective amount of the identified compound and an acceptable carrier. This invention also provides a composition for changing the reproductive behavior of an animal comprising effective amount of the identified compound and an acceptable carrier. This invention provides a

composition for increasing the fertility of a subject comprising effective amount of the identified compound and an acceptable carrier.

5 This invention provides a composition for changing hormonal secretion of a subject comprising effective amount of the identified compound and an acceptable carrier.

10 In an embodiment, the compound is a polypeptide.

This invention provides a transgenic nonhuman living organism expressing DNA encoding a pheromone receptor, either the natural or modified form, and transgenic nonhuman living organism expressing DNA encoding the polypeptide capable of activating or inhibiting the activity of a pheromone receptor.

15
20 This invention also provides a transgenic nonhuman living organism comprising a homologous recombination knockout of the native pheromone receptor.

Brief Description of the Figures

Figure 1. Spatial segregation of the vomeronasal organ
and the main olfactory systems. (A) A
5 drawing of a parasagittal section through the
skull of a rat. The convoluted turbinates of
the main olfactory system (MOE) reside within
the posterior recess of the nasal cavity,
whereas the vomeronasal organ (VNO) resides
10 more anteriorly in a blind-ended pouch within
the septum of the nose. The axons from sensory
neurons of the MOE project to the main
olfactory bulb (OB), whereas the neurons of the
VNO send axons to the anatomically distinct,
15 more posteriorally-placed accessory olfactory
bulb (AOB). (B) A drawing of a coronal
section showing the anatomically distinct
vomeronasal organ and the main olfactory
epithelium. NC, nasal cavity; P, palate.

20 Figure 2. Identification of cDNA clones specifically
expressed in an individual VNO neuron. 20 cDNA
clones initially identified by differential
screening of a cDNA library from a single VNO
neuron were isolated. The inserts were
25 amplified by PCR, electrophoresed on 1% agarose
gels, and blotted to nylon filters. Blots were
annealed with ³²P-labeled cDNA probe from VNO
neuron 1 (A), VNO neuron 2 (B), or a neuron
30 from the main olfactory epithelium (C). Two
cDNA clones (18 and 19) only anneal with cDNA
prepared from VNO neuron 1. One clone (17)
anneals with the cDNA from both VNO neurons,
but not with cDNA from an MOE neuron.

- Figure 3. Expression of VN1 receptor RNA is restricted to
a subset of vomeronasal neurons. Coronal
sections of the vomeronasal organ dissected
from adult male rats were annealed with
digoxigenin-labeled, antisense RNA probes for
(A) the olfactory marker protein (OMP); (B)
The M12 receptor (a receptor expressed in
abundance in the main olfactory epithelium) and
(C) The VNO-specific receptor, VN1. (D) *In
situ* hybridization of VN1 to a coronal section
of turbinates from the newborn main olfactory
epithelium. The arrow in B indicates a single
positive VNO neuron expressing the MOE
receptor, M12. In Panel A, N denotes the
neuroepithelium; L, the lumen of VNO; and V the
vomeronasal vein. In Panel D, the arrow points
to the MOE; NC, nasal cavity. Scale bar equals
120mm
- Figure 4. Deduced amino acid sequences of the pheromone
receptor cDNAs. (A) The deduced amino acid
sequences of seven putative pheromone receptor
cDNAs, VN1, VN2, VN3, VN4, VN5, VN6, VN7 (Seq.
ID. Nos.:8-14) are aligned. Predicted
positions of the seven transmembrane domains
are indicated (I-VII). Amino acid residues
common to at least five of the seven sequences
are highlighted in black. (B) An alignment
between the sequences of the second and third
transmembrane domains of the rat prostaglandin
receptor E3 (rEP3B) (Seq. ID. No.:16), and the
VNO receptor VN2 (Seq. ID. No.:17) showing 28%
identity over this region of the receptor
sequence. (C) An alignment between the
sequences of VN6 (Seq. ID. No.:18) and HG25

(Seq. ID. No.:15) which is deduced from a human clone.

Figure 5. Southern blot analysis with the seven pheromone receptor cDNAs. Rat genomic DNA isolated from liver was digested with PstI (lanes 1) or EcoRI (lanes 2), electrophoresed on 0.8% agarose gels, and blotted to nylon filters. Blots were annealed with ³²P-labeled probes corresponding to the seven different receptor cDNAs, VN1-VN7 (Panels A-G, respectively). Under the high stringency conditions of hybridization and washing used in these experiments, cross hybridization is observed between VN1 and VN2, whereas the other individual receptor probes do not crosshybridize. A mix of six probes specific for each of the six receptor subfamilies (VN2-VN7) was annealed under conditions of high (H) and lower (I) stringency to either PstI (lanes 1), EcoRI (lane 2), or Hind3 (lane 3) cleaved DNA (See Experimental Procedures). Panel I was run separately from Panels A-H, which were electrophoresed on the same gel.

Figure 6. Localization of the individual receptors to distinct subpopulations of cells within the vomeronasal organ. *In situ* hybridization to coronal sections of a dissected VNO using digoxigenin-labeled probes from either the individual receptors, or a mix of the six receptors. Digoxigenin-labeled antisense RNA probes from receptor VN1 (A), receptor VN3 (C), receptor VN4 (D), or a mix of six probes specific for each receptor subfamilies (E) were

annealed to a coronal section of the VNO
dissected from male rats. Panel B shows the
annealing of receptor VN1 probe to a section
through the VNO from a female rat. Panel F
5 shows a high power magnification of (E). VNO
cDNA clones 1-7 label 2.7, 3.8, 1.1, 1.2, 1.1,
1.5, and 3% of the cells in the
neuroepithelium, respectively. The mix of
10 seven probes label 15% of the cells. Scale
bar equals 120 mm.

Figure 7. Receptor expression is restricted to VNO
neurons. A coronal section through the head of
15 an E17 rat shows hybridization of a mix of 6
receptor probes to neurons within the VNO
(arrows), but not to neurons within the main
olfactory epithelium nor to other tissues in
the nose. NC, nasal cavity; S, septum. Scale
bar equals 250 mm. Nucleic acid sequence of cDNA
20 A. The underlined ATG is the initiated codon
used and the underlined TAA is the termination
codon used.

Figure 8. Nucleic acid sequence of VN1 (Seq. ID. No.:2).
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Figure 9. Nucleic acid sequence of VN3 (Seq. ID. No.:3).

Figure 10. Nucleic acid sequence of VN4 (Seq. ID. No.:4).
30 Figure 11. Nucleic acid sequence of VN5 (Seq. ID. No.:5).

Figure 12. Nucleic acid sequence of VN6 (Seq. ID. No.:6).

Figure 13. Nucleic acid sequence of VN7 (Seq. ID. No.:7).
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Figure 14. Nucleic acid sequence of hg25x(Seq. ID. No.:1).

Detailed Description of the Invention

This invention provides an isolated nucleic acid molecule encoding a vertebrate pheromone receptor. In an embodiment, the nucleic acid molecule is a DNA molecule. The DNA may be cDNA, genomic or synthetic DNA. In another embodiment, the nucleic acid is an RNA molecule.

In a further embodiment, the nucleic acid molecule encodes a mammalian pheromone receptor. In a still further embodiment, the nucleic acid molecule encodes a rat pheromone receptor. In another further embodiment, the nucleic acid molecule encodes a human pheromone receptor.

The nucleic acid molecules encoding a pheromone receptor includes molecules coding for polypeptide analogs, fragments or derivatives of antigenic polypeptides which differ from naturally-occurring forms in terms of the identity or location of one or more amino acid residues (deletion analogs containing less than all of the residues specified for the protein, substitution analogs wherein one or more residues specified are replaced by other residues and addition analogs where in one or more amino acid residues is added to a terminal or medial portion of the polypeptides) and which share some or all properties of naturally-occurring forms.

These molecules include but not limited to: the incorporation of codons "preferred" for expression by selected non-mammalian hosts; the provision of sites for cleavage by restriction endonuclease enzymes; and the provision of additional initial, terminal or intermediate sequences that facilitate construction of readily expressed vectors. Accordingly, these changes may result

in a modified pheromone receptor. It is the intent of
this invention to include nucleic acid molecules which
encodes modified pheromone receptor. Also, to facilitate
the expression of receptor in different host cells, it
5 may be necessary to modify the molecule such that the
expressed receptors may reach the surface of the host
cells. The modified pheromone receptor should have
biological activities similar to the unmodified pheromone
receptor. The molecules may also be modified to increase
10 the biological activity of the expressed receptor.

This invention also provides a nucleic acid molecule of
at least 12 nucleotides capable of specifically
hybridizing with a unique sequence within the sequence of
15 the above-described nucleic acid molecule. This nucleic
acid molecule may be DNA or RNA.

This invention provides a vector which comprises the
above-described isolated nucleic acid molecule.

20 In another embodiment, the vector is a plasmid. In a
further embodiment, the plasmid is designated VN1 (ATCC
Accession No.97294). In another embodiment, the plasmid
is designated VN3 (ATCC Accession No. 97295). In a
25 separate embodiment, the plasmid is designated VN4 (ATCC
Accession No.97296). In another embodiment, the plasmid
is designated VN5 (ATCC Accession No.97297). In a
separate embodiment, the plasmid is designated VN6 (ATCC
Accession No.97298). In a still further embodiment, the
30 plasmid is designated VN7 (ATCC Accession No. 97299).

Plasmids VN1, VN3, VN4, VN5, VN6 and VN7 were made by
cloning the DNA inserts which encoding a pheromone
receptor into the XhoI and EcoRI sites of the plasmid
35 pBluescript-. VN1, VN3, VN4, VN5, VN6 and VN7 were

deposited on September 27, 1995 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure. The plasmids, VN1, VN3, VN4, VN5, VN6 and VN7 were accorded ATCC Accession Numbers 97294-97299.

The sequences of the DNA inserts of VN1, VN3, VN4, VN5, VN6 and VN7 were submitted to GenBank and were assigned with accession numbers U36785, U36895, U36896, U36897, U36898, and U36786 respectively. VN2 was assigned with GenBank accession number U36899.

In an embodiment, the above described isolated nucleic acid molecule is operatively linked to a regulatory element.

Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. For example, a bacterial expression vector includes a promoter such as the lac promoter and for transcription initiation the Shine-Dalgarno sequence and the start codon AUG. Similarly, a eukaryotic expression vector includes a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors may be obtained commercially or assembled from the sequences described by methods well-known in the art, for example the methods described above for constructing vectors in general.

This invention provides a host vector system for the production of a polypeptide having the biological

activity of a vertebrate pheromone receptor which comprises the above-described vector and a suitable host.

This invention also provides a host vector system,
5 wherein the suitable host is a bacterial cell, yeast cell, insect cell, or animal cell. The host cell of the above expression system may be selected from the group consisting of the cells where the protein of interest is normally expressed, or foreign cells such as bacterial cells (such as *E. coli*), yeast cells, fungal cells, insect cells, nematode cells, plant or animal cells, where the protein of interest is not normally expressed.
10 Suitable animal cells include, but are not limited to Vero cells, HeLa cells, Cos cells, CV1 cells and various
15 primary mammalian cells.

This invention provides a method of producing a polypeptide having the biological activity of a vertebrate pheromone receptor which comprising growing
20 the above-described host vector system under conditions permitting production of the polypeptide and recovering the polypeptide so produced.

This invention also provides a purified, vertebrate
25 pheromone receptor.

This invention also provides a polypeptide encoded by the above-described isolated vertebrate nucleic acid molecule.
30 This invention provides an antibody capable of binding to a vertebrate pheromone receptor. This invention further provides an antibody capable of competitively inhibiting the binding of the antibody capable of specifically
35 binding to a vertebrate pheromone receptor. In an

embodiment, the antibody is monoclonal. In another embodiment, the antibody is polyclonal.

Monoclonal antibody directed to a pheromone receptor may
5 comprise, for example, a monoclonal antibody directed to
an epitope of a pheromone receptor present on the surface
of a cell. Amino acid sequences may be analyzed by
methods well known to those skilled in the art to
determine whether they produce hydrophobic or hydrophilic
10 regions in the proteins which they build. In the case of
cell membrane proteins, hydrophobic regions are well
known to form the part of the protein that is inserted
into the lipid bilayer which forms the cell membrane,
while hydrophilic regions are located on the cell
15 surface, in an aqueous environment.

Antibodies directed to a pheromone receptor may be
serum-derived or monoclonal and are prepared using
methods well known in the art. For example, monoclonal
20 antibodies are prepared using hybridoma technology by
fusing antibody producing B cells from immunized animals
with myeloma cells and selecting the resulting hybridoma
cell line producing the desired antibody. Cells such as
NIH3T3 cells or 293 cells which express the receptor may
25 be used as immunogens to raise such an antibody.
Alternatively, synthetic peptides may be prepared using
commercially available machines.

As a still further alternative, DNA, such as a cDNA or a
30 fragment thereof, encoding the receptor or a portion of
the receptor may be cloned and expressed. The expressed
polypeptide recovered and used as an immunogen.

The resulting antibodies are useful to detect the
35 presence of pheromone receptors or to inhibit the

function of the receptor in living animals, in humans, or in biological tissues or fluids isolated from animals or humans.

- 5 This antibodies may also be useful for identifying or isolating other pheromone receptor. For example, antibodies against the rat pheromone receptor may be used to screen a human expression library for a human pheromone receptor. Such antibodies may be monoclonal or
- 10 monospecific polyclonal antibody against a selected pheromone receptor. Human expression libraries are readily available and may be made using technologies well-known in the art.
- 15 This invention provides a method for identifying cDNA inserts encoding pheromone receptors comprising: (a) generating a cDNA library which contains clones carrying cDNA inserts from an individual vomeronasal sensory neuron; (b) hybridizing nucleic acid molecules of clones
- 20 from the cDNA libraries generated in step (a) with probes prepared from the individual vomeronasal neuron and probes from a secondary individual vomeronasal neuron or a main olfactory epithelium neuron; (c) selecting clones which hybridized with probes from the individual vomeronasal neuron but not from the second individual vomeronasal neuron or the main olfactory epithelium neuron; and (d) isolating clones which carry the hybridized inserts, thereby identifying the inserts encoding pheromone receptors.
- 25 This invention also provides the above-described method wherein after step (c), further comprising: (a) amplifying the inserts from the selected clones by polymerase chain reaction; (b) hybridizing the amplified
- 30 inserts with probes from the individual vomeronasal
- 35

neuron; and (c) isolating the clones which carry the hybridized inserts, thereby identifying the inserts encoding the pheromone receptors. The probes used may be cDNA .

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This invention also provides cDNA inserts identified by the above methods.

This invention also provides a method for identifying DNA
10 inserts encoding pheromone receptors comprising: (a) generating DNA libraries which contain clones carrying inserts from a sample containing at least one vomeronasal sensory neuron; (b) contacting clones from the cDNA libraries generated in step (a) with nucleic acid
15 molecule of at least 12 nucleotides capable of specifically hybridizing with a unique sequence within the sequence of a pheromone receptor in appropriate conditions permitting the hybridization of the nucleic acid molecules of the clones and the nucleic acid
20 molecule; (c) selecting clones which hybridized with nucleic acid molecule; and (d) isolating the clones which carry the hybridized inserts, thereby identifying the inserts encoding the pheromone receptors. In an embodiment, the sample contain only one individual
25 vomeronasal sensory neuron.

One means of isolating a nucleic acid molecule which encodes a pheromone receptor is to probe a libraries with a natural or artificially designed probes, using methods
30 well known in the art. The probes may be DNA or RNA. The library may be cDNA or genomic DNA.

This invention also provides a method to identify DNA
35 inserts encoding pheromone receptors comprising: (a) generating DNA libraries which contain clones with

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15 inserts from a sample containing at least one vomeronasal sensory neuron; (b) contacting the clones from the DNA libraries generated in step (a) with appropriate polymerase chain reaction primers capable of specifically binding to nucleic acid molecules encoding pheromone receptors in appropriate conditions permitting the amplification of the hybridized inserts by polymerase chain reaction; (c) selecting the amplified inserts; and (d) isolating the amplified inserts, thereby identifying the inserts encoding the pheromone receptors. In an embodiment, the sample contains only one individual vomeronasal sensory neuron. In a separate embodiment of the above methods, the libraries are cDNA libraries. In another embodiment, the libraries are cDNA libraries.
- 15 20 The appropriate polymerase chain reaction primers may be chosen from the conserved regions of the known pheromone receptor sequences. Alternatively, the primers may be chosen from the regions which are the active sites for the binding of ligands.
- This invention also provides DNA inserts identified by the above methods.
- 25 30 35 This invention provides a method to isolate DNA molecules encoding pheromone receptors comprising: (a) contacting a biological sample known to contain nucleic acids with appropriate polymerase chain reaction primers capable of specifically binding to nucleic acid molecules encoding pheromone receptors in appropriate conditions permitting the amplification of the hybridized molecules by polymerase chain reaction; (b) isolating the amplified molecules, thereby identifying the DNA molecules encoding the pheromone receptors. In an embodiment, the sample contains DNA. In another embodiment, the sample contains

genomic DNA.

This invention also provides the nucleic acid molecules isolated by the above method.

5

This invention provides a method of transforming cells which comprises transfecting a host cell with a suitable vector comprising a nucleic acid molecule encoding a pheromone receptor as described above. This invention 10 also provides transformed cells produced by above method.

10

The invention also provides transformed cells wherein the host cells are not usually expressing pheromone receptors and transformed cells wherein the host cells are 15 expressing pheromone receptors.

20

This invention provides a method of identifying a compound capable of specifically binding to a vertebrate pheromone receptor which comprises contacting a transfected cells or membrane fractions of the above transfected cells with an appropriate amount of the compound under conditions permitting binding of the compound to such receptor, detecting the presence of any such compound specifically bound to the receptor, and thereby determining whether the compound specifically binds to the receptor.

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This invention provides a method of identifying a compound capable of specifically binding to a vertebrate pheromone receptor which comprises contacting an appropriate amount of the purified pheromone receptor with an appropriate amount of the compound under conditions permitting binding of the compound to such purified receptor, detecting the presence of any such compound specifically bound to the receptor, and thereby 35

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determining whether the compound specifically binds to the receptor.

In an embodiment, the purified receptor is embedded in a lipid bilayer. The purified receptor may be embedded in the liposomes with proper orientation to carry out normal functions. Liposome technology is well-known in the art.

This invention also provides a method of identifying a compound capable of activating the activity of a pheromone receptor which comprises contacting the transfected cells or membrane fractions of the above described transfected cells with the compound under conditions permitting the activation of a functional pheromone receptor response, the activation of the receptor indicating that the compound is capable of activating the activity of a pheromone receptor.

This invention provides a method of identifying a compound capable of activating the activity of a pheromone receptor which comprises contacting a purified pheromone receptor with the compound under conditions permitting the activation of a functional pheromone receptor response, the activation of the receptor indicating that the compound is capable of activating the activity of a pheromone receptor. In an embodiment, the purified receptor is embedded in a lipid bilayer. As discussed hereinabove, the purified receptors may be embedded in liposomes with proper orientations to carry out their normal functions.

This invention provides a method of identifying a compound capable of inhibiting the activity of a pheromone receptor which comprises contacting the transfected cells or membrane fractions of the above

described transfected cells with an appropriate amount of the compound under conditions permitting the inhibition of a functional pheromone receptor response, the inhibition of the receptor response indicating that the compound is capable of inhibiting the activity of a pheromone receptor.

- This invention provides a method of identifying a compound capable of inhibiting the activity of a pheromone receptor which comprises contacting an appropriate amount of the purified pheromone receptor with an appropriated amount of the compound under conditions permitting the inhibition of a functional pheromone receptor response, the inhibition of the receptor response indicating that the compound is capable of activating the activity of a pheromone receptor. In an embodiment of the above method, the purified receptor is embedded in a lipid bilayer.
- In another embodiment of the above methods, the compound used is not previously known.

This invention also provides compounds identified by the above methods. This invention further provides a pharmaceutical composition comprising an effective amount of the identified compound and a pharmaceutically acceptable carrier.

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Pharmaceutically acceptable carriers are well known to those skilled in the art and include, but are not limited to, 0.01-0.1M and preferably 0.05M phosphate buffer or 0.8% saline. Additionally, such pharmaceutically acceptable carriers may be aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene

glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media.

- 5 Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like.
- 10 Preservatives and other additives may also be present, such as, for example, antimicrobials, antioxidants, chelating agents, inert gases and the like.

This invention provides a method for manipulating the
15 maternal behavior of a female subject comprising administering effective amount of the above compound to the female subject. In an embodiment, the female subject is a human. In another embodiment, the subject is an animal.

20 This invention provides a method for manipulating the social behavior of a subject comprising administering effective amount of the above compound to the subject. In an embodiment, the subject is a human. In another
25 embodiment, the subject is an animal

This invention provides a method for manipulating the reproductive functions of a subject comprising administering effective amount of the above compound to
30 the subject.

This invention also provide a method for manipulating the reproductive behavior of a subject comprising administering effective amount of the above compound to
35 the subject.

This invention provides a method for increasing the fertility of a subject comprising administering effective amount of the above compound to the subject.

5 This invention provides a method for manipulating hormonal secretion of a subject comprising administering effective amount of the above compound to the subject. In an embodiment, the hormone is the luteinizing hormone release hormone. In another embodiment, the hormone is
10 the luteinizing hormone. In a further embodiment, the hormone is the prolactin release hormone. In a still further embodiment, the hormone is the prolactin.

15 This invention also provides a method for changing food intake rate of a subject comprising administering effective amount of the above compound to the subject.

20 The above methods of different uses of the identified compounds are applicable to different animals as well as human.

25 This invention provides a composition for manipulating the maternal behavior of a female subject comprising effective amount of the above compound and an acceptable carrier. This invention also provides a composition for manipulating the social behavior of a subject comprising effective amount of the above compound and an acceptable carrier. This invention further provides a composition for manipulating the reproductive functions of a subject comprising effective amount of the above compound. This invention also provides a composition for changing the reproductive behavior of an animal comprising effective amount of the above compound. This invention provides a composition for increasing the fertility of an animal comprising effective amount of the above compound and an
30
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acceptable carrier. The subject may be human or animal.

This invention provides a composition for manipulating
hormonal secretion of a subject comprising effective
amount of the above compound and an acceptable carrier.
In an embodiment, the hormone is the luteinizing hormone
release hormone. In another embodiment, the hormone is
the luteinizing hormone. In a further embodiment, the
hormone is the prolactin release hormone. In a still
further embodiment, the hormone is the prolactin. The
subject may be human or animal.

As it is well known in the art, various carriers may be used according to this invention. For example, the compound may dissolve in physiological saline for administration to animal or human.

In an embodiment, the compound is a polypeptide.

20 This invention provides a transgenic nonhuman living organism expressing DNA encoding a vertebrate pheromone receptor. This invention also provides a transgenic nonhuman living organism expressing DNA encoding the polypeptide which is capable of inhibiting the activity 25 of a pheromone receptor. In an embodiment, the living organism is a transgenic animal.

This invention also provides a transgenic nonhuman living organism comprising a homologous recombination knockout of the native pheromone receptor. In an embodiment, the transgenic is an animal.

One means available for producing a transgenic animal, with a mouse as an example, is as follows: Female mice are mated, and the resulting fertilized eggs are

dissected out of their oviducts. The eggs are stored in an appropriate medium such as M2 medium (Hogan B. et al. Manipulating the Mouse Embryo, A Laboratory Manual, Cold Spring Harbor Laboratory (1986)). DNA or cDNA encoding 5 a pheromone receptor is purified from a vector by methods well known in the art. Inducible promoters may be fused with the coding region of the DNA to provide an experimental means to regulate expression of the trans-gene. Alternatively or in addition, tissue specific regulatory elements may be fused with the coding region 10 to permit tissue-specific expression of the trans-gene. The DNA, in an appropriately buffered solution, is put into a microinjection needle (which may be made from capillary tubing using a pipet puller) and the egg to be injected is put in a depression slide. The needle is 15 inserted into the pronucleus of the egg, and the DNA solution is injected. The injected egg is then transferred into the oviduct of a pseudopregnant mouse (a mouse stimulated by the appropriate hormones to maintain pregnancy but which is not actually pregnant), where it proceeds to the uterus, implants, and develops to term. As noted above, microinjection is not the only method for 20 inserting DNA into the egg cell, and is used here only for exemplary purposes.

25 This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of 30 the invention as described more fully in the claims which follow thereafter.

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Experimental Details

Experimental Strategy

Applicants' initial efforts to identify the genes encoding the pheromone receptors were based upon the assumption that the main olfactory epithelium and the vomeronasal organ might share a common evolutionary origin such that DNA sequence homology may exist between the two receptor families. However, low stringency hybridization of MOE receptor probes to rat vomeronasal cDNA libraries, as well as polymerase chain reactions (PCR) using conserved motifs from both the family of odorant receptor genes, as well as from the superfamily of known seven transmembrane domain receptors were consistently unsuccessful. Moreover, the components of the olfactory signal transduction cascade in the main olfactory epithelium [(the olfactory-specific G-protein, G_{olf} , (Jones and Reed, 1989), the olfactory-specific adenylate cyclase (Bakalyar and Reed, 1990), and the cyclic nucleotide responsive ion channel (Dhallan et al., 1990; Ludwig et al., 1990)] were not detectable in VNO neurons by *in situ* hybridization or by screening cDNA libraries (data not shown). These observations suggested that the pheromone receptors and the signal transduction pathways which they activate might have evolved independently in the VNO and the MOE.

Applicants therefore developed a cloning procedure that made no assumptions concerning the structural class of the receptor molecules. Rather, applicants only assumed that the expression of the pheromone receptors would be restricted to the vomeronasal organ, and that individual neurons within the VNO were likely to express different receptor genes. In the main olfactory epithelium, about 1% of the mRNA in a given sensory cell encodes a given receptor (Vassar et al., 1994). However, the thousand

different receptor genes are each expressed in different neurons such that the frequency of a specific receptor RNA will be diluted to 0.001% of the mRNA message population. The generation of libraries from individual neurons provided an experimental solution to the problem of detecting a specific mRNA in a heterogeneous population of neurons. RT-PCR was therefore used to generate double-stranded cDNA, as well as cDNA libraries from individual vomeronasal sensory neurons. Applicants expected that the frequency of a specific receptor cDNA in libraries from single neurons would be about 1%. Differential screening of such libraries from single neurons should therefore permit the isolation of pheromone receptor genes.

In control experiments, the cDNA library prepared from a single rat VNO neuron was screened with probes for tubulin and olfactory marker protein (OMP) to determine whether these libraries accurately represent the mRNA population. The frequency of these clones suggested that the representation of a given RNA was not biased in the construction of the library (see Experimental Procedures). One thousand recombinant phages from a cDNA library prepared from VNO neuron 1 were then screened in triplicate with cDNA probes prepared from VNO neuron 1, a second VNO neuron (VNO neuron 2), and a neuron from the MOE. About 2% of the cDNA clones screened showed specific hybridization with cDNA probes from VNO neuron 1, but not with probes from VNO neuron 2 or the MOE neuron. The specificity of these cDNA clones was further examined in a more sensitive assay. The inserts from these cDNA clones were amplified by PCR and the DNA products were hybridized on Southern blots with cDNA probes from VNO neuron 1, VNO neuron 2, or from an MOE sensory neuron (Figure 2). Of 20 clones initially

isolated from the VNO neuron 1 cDNA library, only two (clones 18 and 19 in figure 2A) appeared to be specific to VNO neuron 1 in this more sensitive screen. These two clones represented independent isolates of an identical 5 cDNA sequence present within the cDNA library of VNO neuron 1 at a frequency of 0.5%. This cDNA was used as a probe to isolate full-length clones from a cDNA library with larger inserts constructed with RNA prepared from several dissected vomeronasal organs. A full length 10 clone, VN1 encodes a seven transmembrane domain receptor (see below).

The pattern of expression of this cDNA was determined by 15 performing RNA *in situ* hybridization to sections through the rat vomeronasal organ. In cross section, a thick multicellular sensory epithelium lines half of the lumen of the vomeronasal organ (Figure 3). *In situ* hybridization demonstrates that mature VNO neurons uniformly express the olfactory marker protein (OMP) 20 (Figure 3A). In contrast, the cDNA specific for VNO neuron 1 localized to a subpopulation of VNO neurons (Figure 3C). No hybridization was observed in the MOE (Figure 3D), or in any other neural or non-neural cells (see below).

25 Thus, difference cloning from libraries prepared from single neurons has allowed the isolation of a novel seven transmembrane domain receptor expressed in VNO sensory neurons.

30 **The Sequence of Several Members of the Receptor Gene Family**

Applicants observed that VN1 is expressed in about 4% of 35 the vomeronasal sensory neurons. This suggested the existence of a gene family with individual member genes

expressed in different subsets of neurons. Applicants therefore used both PCR and high and low stringency hybridization to VNO cDNA libraries to identify possible members of a receptor gene family expressed in other VNO neurons (see Experimental Procedures). The sequences of seven different cDNAs obtained in this manner are aligned in Figure 4. Hydropathy analysis suggests that each of the seven sequences contain seven hydrophobic stretches that represent potential transmembrane domains. Sequence analysis suggests that these putative receptors are likely to adopt a structure similar to that of the previously characterized superfamily of seven transmembrane receptors. However, the VNO receptors do not share any of the conserved sequence motifs exhibited by members of the previously identified superfamily (Baldwin, 1993; Probst et al., 1992). One region of homology however is observed with the family of mammalian prostaglandin receptors throughout the second and third transmembrane domains (Figure 4B). Twenty-five percent identity is observed between VN2 and the rat E3 prostaglandin receptor over these two domains, but no significant sequence homology is observed in other regions of the molecule. Prostaglandins are potent pheromones eliciting mating in fish, but their role as mammalian pheromones is unknown. However, this level of homology over a small region of the protein does not permit us to argue that the receptors may recognize prostaglandins.

Overall, the seven VNO cDNA sequences share between 47% and 87% sequence identity. As observed previously for the odorant receptors from the MOE (Buck and Axel 1991), this family of VNO receptors exhibits significant divergence within the transmembrane domains, the presumed site of ligand binding (Strader et al., 1994). This

pattern of divergence suggests that the different members may permit the binding of different structural classes of ligands.

5 **The Size of the Gene Family**

Applicants have analyzed the size of the vomeronasal receptor gene family by performing hybridizations to genomic DNA, as well as quantitative screening of genomic libraries. The seven cDNAs that applicants have characterized fall within six subfamilies as defined by the observation that no crosshybridization is observed among the different subfamilies under high stringency conditions. cDNA probes from each of the six subfamilies were then annealed to Southern blots of rat genomic DNA after digestion with two different restriction endonucleases (Figure 5). The vomeronasal receptor genes analyzed thus far do not contain introns within the coding region (data not shown). Restriction cleavage was performed with endonucleases that do not cleave within the cDNAs applicants have isolated such that the number of hybridizing bands will closely approximate the number of receptor genes. Probes from each of the subfamilies identified from two to eight bands in genomic DNA such that a total of about 20 bands were detected in hybridizations with the six individual probes. A mix of six probes identifies about 20 bands in genomic DNA at high stringency of hybridization (Figure 5H) and more than 30 bands using less stringent conditions (Figure 5I).

30 An independent estimate of the size of the gene family was obtained by screening a genomic library. A mix of the seven cDNA clones was used as a hybridization probe under reduced stringency conditions to identify about 35 positive clones per haploid genome. Thus, the data from

Southern blotting and screens of genomic library are in accord with one another and indicate that the multigene family of vomeronasal receptors applicants have identified consists of between 30 and 40 genes.

5

The Pattern of Receptor Expression in the Vomeronasal Organ

Applicants performed *in situ* hybridization to examine the spatial pattern of receptor expression in the sensory epithelium of the VNO. The VNO consists of a blind-ended tubular structure which extends in an anterior posterior dimension within the septum . In cross section, the sensory eipithelium lines the medial half of the tube and a vein surrounded by non-neuronal tissue resides more laterally (Figure 3A, and Figure 6). RNA *in situ* hybridization experiments were performed with digoxigenin-labeled RNA antisense probes from each of the six subfamilies under high stringency conditions, such that it was likely that a given probe will only detect members within its own subfamily. The results with each of the six probes were qualitatively indistinguishable. In each case, applicants observed a punctate distribution of cells expressing a given receptor RNA (Figure 6). No differences in the patterns of *in situ* hybridization were observed between males and females (Figure 6A and B) Each probe detected from about 1-4% of the VNO sensory neurons. These data contrast with hybridization patterns observed with the probe for the olfactory marker protein OMP (Figure 3A), which demonstrated uniform labeling of the VNO epithelium. Control sections hybridized with sense receptor probes revealed no specific signal (data not shown). Expression of this gene family was only observed in VNO neurons, no labelling was observed in the sensory neurons of the MOE (Figures 3D and 7). Hybridization of the M12 receptor from the MOE reveals a

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rare positive cell at a frequency of about 1 in 20,000 VNO neurons (Figure 3B). Finally, no expression of the VNO receptors was observed upon *in situ* hybridization to sections through brain, kidney, testes, and liver (data 5 not shown).

Analysis of several sections through the entire VNO suggested that neurons expressing a given receptor are not topologically localized but rather are randomly 10 distributed along the anterior-posterior axis. In cross section, however, neurons expressing the receptor family applicants have cloned are preferentially localized to the apical two thirds of the zone of OMP positive cells. Previous studies in the opossum have demonstrated that 15 this apical zone of neurons expresses the G protein G_{i2a} , whereas the more basal zone expresses G_o (Halpern et al., 1995), and a similar pattern is observed in rat (Belluscio, L., Dulac, C., and Axel, R. unpublished 20 studies). Therefore, the expression of the family of receptors applicants have isolated may be restricted to G_{i2a} positive cells. It is possible that the G_o positive cells express a more distant family of receptors.

25 **Individual Neurons Express Different Complements of Receptors.**

In the main olfactory epithelium, a given neuron is likely to express only one receptor from the family of 30 one thousand receptor genes. Moreover, neurons expressing a given receptor project their axons to one or a small number of topographically defined glomeruli within the olfactory bulb. The regulated expression of odorant receptors assuring that only one receptor is expressed in individual olfactory neurons is an important element in the coding of olfactory information in the 35 main olfactory system. Quantitative analysis of the in

situ hybridizations of the VNO receptor probes indicate that neurons within the VNO similarly express only a single receptor gene.

- 5 The observation that 1-4% of the VNO neurons express a
given receptor subfamily suggests that each cell
expresses only a subset of receptor genes. If applicants
demonstrate that each of the different receptor probes
hybridizes with distinct non-overlapping subpopulations
of neurons, this would provide evidence that neurons
differ with respect to the receptors they express.
10 Sections were annealed with probes specific for each of
the six receptor subfamilies, either individually or with
a mixture of six probes (Figure 6). If each receptor is
expressed in a distinct non-overlapping subpopulation of
neurons, then the sum of the cells identified with the
15 six probes should equal the number of cells identified
with the mixed probe. In accord with this suggestion,
applicants observe that the percentage of olfactory
neurons detected with the mixed probe (15%) is
20 significantly greater than the percentage detected with
any of the individual probes alone, and approximates the
sum of the percentage of positive neurons detected with
the six individual probes (12%). These values are
25 present in the legend to Figure 6. These results suggest
that the six receptor subfamilies are expressed in
distinct non-overlapping populations of olfactory neurons
and provide support for a model in which a single sensory
neuron expresses a single receptor gene.

Experimental Discussion

Applicants have identified a novel family of seven transmembrane domain proteins which encode the mammalian pheromone receptors. Differential screening of cDNA libraries constructed from single sensory neurons

initially led to the isolation of a family of putative receptor genes. Each member of the gene family is expressed in a small subpopulation of neurons such that the seven putative receptor genes applicants have cloned identify 15% of the cells in the VNO. The expression of this gene family is restricted to neurons within the VNO and is not observed in sensory neurons of the MOE nor in other non-neuronal cells. This array of properties is consistent with those predicted for the mammalian pheromone receptors. Proof that these sequences indeed encode pheromone receptors will require the demonstration that these receptor proteins bind pheromones and are able to transduce pheromone binding into alterations in membrane potential.

The experimental approach employed to isolate this gene family, differential screening of a cDNA library constructed from a single neuron, may be more broadly applicable to the analysis of the specific gene expression in diverse populations of cells. In the nervous system, for example, functionally distinct neurons each expressing different genes, and each projecting to different targets, are often interspersed. It has therefore been difficult to isolate RNA species unique to functionally distinct subsets of neurons within a heterogeneous cell population. The ability to generate cDNA libraries from individual cells within a diverse population of neurons may permit the identification of that subset of genes which afford a cell a unique identity.

How Large is the Gene Family?

The number of receptor genes expressed in the two distinct olfactory organs of mammals is likely to reflect the repertoire of odors recognized by the two populations

of olfactory sensory neurons. The main olfactory organ can recognize a universe of odors which define an organism's environment, whereas the VNO largely recognizes molecules distinctive to the species that define the reproductive and social status of individuals within any given species. Olfactory receptors of the MOE is encoded by a family of about 1000 genes (Buck and Axel, 1991; Parmentier et al., 1992; Ben Arie et al., 1994). Since the range of molecules detected by the VNO is thought to be far smaller than the odors detected by the MOE, applicants anticipated that the repertoire of pheromone receptors would be far smaller as well. Gene cloning and Southern blotting with genomic DNA provide an estimate of the size of the pheromone receptor repertoire. A screen of genomic libraries with a mix of probes detect approximately 35 positive clones per genome. This value is in accord with the results of genomic blot hybridization at low stringency which identifies about 30 discrete genes with the available probes. This minimum estimate of 30-35 genes clearly provides a lower limit of the size of the VNO receptor repertoire since it is likely that the seven genes applicants have cloned do not allow us to detect all the members of the pheromone receptor gene family.

In situ hybridization experiments with individual probes provide an independent estimate of the number of receptor genes expressed in the VNO. Each of the seven putative pheromone receptor genes labels about 1-4% of the vomeronasal sensory neurons, whereas a mix of the genes representing the six subfamilies detects about 15% of the VNO neurons. These data suggest that a given neuron expresses only one pheromone receptor gene. Since the six subfamily probes detect about 20 genes in the chromosome at high stringency, and label 15% of the VNO

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neurons, applicants estimate that the repertoire of pheromone receptors may consist of about 100 distinct genes.

5 **The Relationship Between the Two Olfactory Organs**

The sequences of the odorant receptors of the MOE and the pheromone receptors of the VNO share no apparent homology, indicating that the two olfactory sensory systems of mammals have evolved independently. This 10 suggestion is in accord with the observation that the signal transduction machinery of the MOE cannot be detected in the neurons of the VNO. What is the evolutionary origin of the VNO? Pheromone-responsive 15 neurons and neurons responsive to the more general class of odorants are likely to have been present throughout vertebrate evolution. With the emergence of terrestrial forms, segregation of the two types of neurons may have occurred, generating a distinct vomeronasal organ that facilitates the access and binding of the two classes of 20 odorous ligand. Thus, terrestrial vertebrates from amphibians to mammals, including humans, retain two distinct olfactory systems, the VNO and the MOE (Bertmar, 1981; Eisthen, 1992; Potiquet, 1891; Stensaas et al., 1991; Moran et al., 1991; Garcia-Velasco and Mondragon, 25 1991).

These two functional classes of sensory neurons are also apparent in invertebrate olfactory systems. These 30 observations immediately pose the question as to whether homologs of the two different families of vertebrate olfactory receptors are present within the genome of invertebrates. Attempts to identify genes related to the large family of MOE receptors in *C. Elegans* (Bargmann, personal communication) and *Drosophila* (Amrein, H., 35 Vosshall, L., and Axel, R., unpublished studies;

Carlson, J., personal communication) have thus far been unsuccessful. Several large families of seven transmembrane receptor genes expressed in subsets of *C. elegans* chemosensory neurons have recently been
5 identified (Troemel et al., 1995) However, these sequences share no homology with the mammalian receptor sequences from either the VNO or MOE. It is possible that the identification of additional families of receptors will reveal a common evolutionary ancestor to
10 the vertebrate and invertebrate olfactory systems. Alternatively, the differences in the chemical nature of the odorants and differences in the physiological consequences of odor recognition might suggest independent origins for the invertebrate and vertebrate
15 olfactory system.

The Logic of Olfactory Coding in the MOE and VNO

Analysis of the patterns of expression of receptor genes in the main olfactory system has provided significant
20 insight into mechanisms for the diversity and specificity of odor recognition in mammals. Similarly, the isolation of the pheromone receptors from the vomeronasal organ is likely to help to elucidate the logic of olfactory perception in the vomeronasal system. The initial step
25 in olfactory discrimination by the MOE requires the interaction of odorous ligands with one of the multiple seven transmembrane domain receptors on olfactory sensory neurons. Discrimination among odorants requires that the brain determine which of numerous receptors has been activated.
30 Since individual olfactory sensory neurons in the MOE are likely to express only a single receptor gene, the problem of distinguishing which receptors have been activated reduces to a problem of distinguishing which neurons have been activated.

Recent experiments demonstrate that neurons expressing a given receptor, and therefore responsive to a given odorant, project their axons to one or a small number of discrete loci or glomeruli within the olfactory bulb
5 (Vassar et al., 1994; Ressler et al. 1994; Mombaerts et al., unpublished). The positions of specific glomeruli are topographically fixed, and are conserved in the brains of all animals within a species. These data provide physical evidence that the olfactory bulb
10 provides a two-dimensional map that identifies which of the numerous receptors have been activated within the sensory epithelium. Such a model is in accord with previous experiments demonstrating that different odors elicit spatially defined patterns of glomerular activity
15 in the olfactory bulb (Kauer et al., 1987; Stewart et al., 1979; Lancet et al., 1982; Mori et al., 1992; Imamura et al., 1992; Katch et al., 1993). Thus, the quality of an olfactory stimulus would therefore be encoded by the specific combination of glomeruli
20 activated by a given odorant.

At one level, the vomeronasal system shares anatomic and physiologic features with the main olfactory system, suggesting that similar experiments with pheromone receptors might also provide insight as to how the recognition of odors by the VNO leads to the elaboration of innate behaviors. Primary olfactory sensory neurons within the vomeronasal organ project a single unbranched axon which then synapses with dendrites of mitral cells
25 in the accessory olfactory bulb, the first relay station for vomeronasal signalling in the brain. At a molecular level, applicants have identified a family of pheromone receptor genes that encode seven transmembrane domain proteins. Individual VNO neurons are likely to express
30 only a single receptor gene. Cells expressing a specific
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receptor are randomly dispersed within the apical zone of the sensory epithelium. Thus, the pattern of pheromone receptor expression shares striking similarities with the expression of odorant receptors in the MOE.

5 At first glance, the anatomy and molecular organization of the VNO and MOE as well as that of the main and accessory olfactory bulb appear quite similar. There are, however, important differences. In the MOE, the
10 mitral cells, the major output neurons of the olfactory bulb, project a primary dendrite to a single glomerulus suggesting a one-to-one correspondence between mitral cell and sensory axon, such that a given mitral cell can respond to the activation of only a single class of
15 sensory neurons. The task of discerning which sensory neurons have been activated must therefore be accomplished by integration at higher cortical centers. Mitral cells of the accessory bulb, however, exhibit a more complex primary dendritic array allowing synapse formation with more than one glomerulus and therefore
20 more than one class of sensory neurons (Macrides et al., 1985; Takami and Graziadei, 1991). These observations suggest that in the vomeronasal system, integration permitting the detection of a specific combination of
25 different receptors activated by pheromones may occur in the accessory olfactory bulb.

The VNO and the main olfactory system reveal striking differences in the secondary projections to the cortex and in the responses elicited by the two sensory systems.
30 VNO neurons project directly to the amygdala and hypothalamus leading to innate and stereotypic behavioral responses (Broadwell, 1975; Scalia and Winans, 1975; Winans and Scalia, 1970; Kevetter and Winans, 1981;
35 Krettek and Price, 1977; 1978a). In contrast, the

- projections from the main olfactory organ activate higher cortical centers resulting in a measured emotional or cognitive response. The projections from the vomeronasal system to the hypothalamus also control the release of 5 luteinizing hormone release hormone (LHRH) and prolactin release hormone (PRF), increasing LH and prolactin levels both centrally and peripherally (reviewed in Keverne, 1983, Meredith and Fernandez-Fewell, 1994). In this manner, stimulation of the vomeronasal system can 10 coordinate the activation of central neural pathways with dramatic neuroendocrine changes to elicit a characteristic array of innate reproductive and social behaviors.
- 15 The coding of olfactory information is likely to be far simpler in the vomeronasal system than in the main olfactory pathway. The receptor repertoire in the VNO is an order of magnitude smaller than in the MOE. Moreover, 20 integration in the vomeronasal pathway is apparent in the accessory bulb and the secondary projections synapse on small number of loci in the amygdala. This is in sharp contrast to the complexity of higher cortical pathways required for processing olfactory information from the MOE. Thus, the vomeronasal system may permit the 25 analysis of the molecular events which translate the bindings of pheromones into innate stereotypic behaviors.

Pheromone Receptors in Humans

Until recently, the VNO in humans was thought to be an 30 atretic organ of vestigial function. Recent reports, however, identify a structurally intact vomeronasal organ in virtually all biopsy specimens examined (Moran, et al., 1991; Stensaas, et al., 1991, Garcia-Velasco and Mondragon 1991) Activation of neurons has been observed 35 in the human VNO in response to purified components from

skin extracts (Monti-Bloch, et al, 1994), but the physiological or behavioral consequences of VNO activation remain elusive. Moreover, it has been difficult to identify human pheromones that elicit innate behavioral arrays since behavior in humans is far more likely to be tempered by learning and experience.

In preliminary experiments, applicants have identified homologs of the rodent VNO receptors in human genomic DNA. Low stringency screens of a human genomic library with a mix of rat VNO receptor cDNAs identifies human homologs at a frequency of about 15 per haploid genome. Partial sequence of two clones reveals 41% and 48% identity with the closest rat homologs (Figure 4C; Sequence ID. No.:15; The human amino acid sequence is designated as HG25). The nucleic acid sequence of a clone, hg25X, is presented in Figure 14 with Sequence ID. No.:7. Even though both genomic clones reveal stop codons within the coding region indicating that these two human sequences are pseudogenes, these clones provide useful tools to obtain clones which code for a functional VNO receptors. The identification of putative pheromone receptors may provide insight into the chemical nature of the pheromones, the mechanisms by which the perception of pheromones lead to innate behaviors and the possible role of this sensory system in humans.

Experimental Procedures

30 Preparation and Screening of Single Cell cDNA Libraries

The main olfactory epithelium (MOE) and vomeronasal organs (VNO) were dissected from adult Sprague-Dawley rats. The synthesis and amplification of single cell cDNA was performed according to Brady et al., 1990 with

modifications. Small pieces of tissue were dissociated for 10' at 37°C in phosphate buffered saline (PBS) (without Ca²⁺, Mg²⁺) 0.025% trypsin, 0.75mM EDTA. After gentle trituration of the tissues in Dulbecco's modified Eagle's medium plus 10% calf serum, cells were collected by centrifugation and resuspended in ice cold PBS. The cell suspension was observed on a Leitz inverted microscope and olfactory sensory neurons were identified as bipolar neurons with an axonal process and a dendrite terminating in an olfactory knob. Isolated neurons were picked with a Leitz micromanipulator fitted with a pulled and beveled microcapillary. Single cells were seeded in thin-walled PCR reaction tubes (Perkin Elmer) containing 4μl of ice cold cell lysis buffer (50mM Tris-HCl (pH8.3), 75mM KCl, 3mM MgCl₂, 0.5% NP-40, containing 80ng/ml pd(T)₁₉₋₂₄ (Pharmacia), 5u/ml Prime Rnase Inhibitor (5'-3' Inc.), 324u/ml RNAGuard (Pharmacia) and 10μM each of dATP, dCTP, dGTP and dTTP). Lysis was subsequently performed for 1' at 65°C. First strand cDNA synthesis was then initiated by adding 50u Moloney murine leukemia virus- and 0.5u avian-reverse transcriptases (BRL) followed by incubation for 10' at 37°. Samples were heat-inactivated for 10' at 65° C and a poly(A) was added to the first strand cDNA product by adding an equal volume of 200mM potassium cacodylate pH7.2, 4mm CoCl₂, 0.4mM DTT, 200μM dATP containing 10 units terminal transferase (Boehringer) for 15' at 37° C. Samples were heat-inactivated 10' at 65°C and the contents of each tube was brought to 100μl with a solution made of 10mM TrisHCl (pH8.3), 50mM KCl, 2.5mM MgCl₂, 100μg/ml bovine serum albumin, 0.05% Triton X-100 and containing 1mM of dATP, dCTP, dGTP, dTTP, 10 units Taq polymerase (Perkin Elmer) and 5μg of the PCR primer AL1. The AL1 sequence is ATT GGA TCC AGG CCG CTC TGG ACA AAA TAT GAA TTC (T)₂₄. PCR amplification was then performed according to the

following schedule : 94°C for 1', 42°C for 2' and 72°C for 6' with 10" extension per cycle for 25 cycles. Five additional units of Taq polymerase were then added before performing 25 more cycles. In this manner, PCR amplified cDNA was synthesized from RNA of individual neurons.

Aliquots of single cell cDNA were run on 1% agarose gels, blotted on nylon membrane (Hybond N+, Amersham) and hybridized with several DNA probes to determine the representation of specific sequences in amplified cDNA. The probes included highly expressed genes (tubulin, OMP), gene expressed at lower level (Go) as well as genes whose expression is restricted to either MOE (G_{olf}) or VNO (G_{i2a}) neuron. The relative level of these genes in amplified cDNA prepared from individual neurons is in accord with levels determined by either *in situ* hybridization or screening more classical cDNA libraries. These data suggest that the amplified cDNA from individual neurons contains an accurate representation of sequences in mRNA.

One microgram of the cDNA prepared from VNO neuron 1 was purified by phenol/chloroform extraction, digested with EcoR1, ligated into EcoR1 predigested and dephosphorylated λZAPII phage arms (Stratagene) and packaged according to standard procedures. The library prepared from VNO neuron 1 consisted of 5×10^4 pfus with an average insert size of 600bp. The frequency of OMP and tubulin positive plaques (0.2%) suggested that the representation of a given RNA was not biased during the construction of the library.

Amplified cDNA from single cells was used as probe by reamplifying 1 μ l of neuron cDNA for 10 cycles with the AL1 primer in the presence of 100 μ Ci of 32P-dCTP. One

thousand recombinant phages from VNO neuron 1 library were plated at low density and triplicate filters (Hybond N+, Amersham) were prehybridized at 65°C in 0.5M sodium phosphate buffer (pH7.3) containing 1% bovine serum albumin and 4% SDS. Hybridization was carried out in the same buffer and at 65° C after adding 10⁷cpm/ml of the amplified cDNA probe made from either VNO neuron 1, VNO neuron 2 or from a MOE neuron. Filters were washed three times at 65°C in 0.5% SDS and 0.5X SSC. Twenty phage plaques showing specific hybridization with the VNO neuron 1 probe were isolated. Phage inserts were amplified by PCR, run on 1% agarose gels, transferred to nylon membranes and were again hybridized with single cell cDNA probes as described above. Phages 18 and 19 contained cDNA inserts which appeared to hybridize only to VNO neuron 1 cDNA probe. Plasmids were obtained from the isolated phages by performing phagemid rescue as instructed by the manufacturer (Stratagene). DNA sequence analysis was performed on plasmid DNAs using the Sequenase system (United States Biochemical Corp).

Isolation and Analysis of Full-length cDNA Clones

Poly A+ RNA was isolated from VNOs dissected from adult male or female rats using the polyA+ isolation kit (Stratagene) according to the manufacturers instructions. cDNA libraries were prepared in the λZapII vector (Stratagene) according to standard procedures (Sambrook et al., 1989). 2 X 10⁵ independent recombinant phages from the male and female VNO cDNA libraries were screened under high stringency hybridization (68°C in 0.5M sodium phosphate buffer (pH7.3) containing 1% bovine serum albumin and 4% SDS) with a ³²P- labelled probe (Prime-it, Stratagene) prepared from the VNO neuron 1 specific clone 18. This allowed the isolation of two

full length cDNA clones, VN1 and VN2. In further screens one additional crosshybridizing cDNA clone, VN3, was obtained by low stringency hybridization (55°C in the same buffer as described above) of a mix of VN1 and VN2 probes to the VNO cDNA libraries. Conserved motifs within these cDNA clones were used to generate PCR primers which were then used to amplify additional sequences from the VNO cDNA libraries. This PCR product, along with the three cDNA clones were used as probes in further hybridizations to obtain four additional full-length cDNAs.

Southern Blotting and *In Situ* Hybridization Analysis

Genomic DNA prepared from Sprague-Dawley rat liver was digested with the restriction enzymes EcoR1, Pst1 or Hind3, size fractionated on 0.8% agarose gels, and blotted onto nylon membrane (Sambrook et al., 1989). The membranes were cross-linked under UV light, prehybridized and hybridized in 0.5M sodium phosphate buffer (pH7.3) containing 1% bovine serum albumin and 4% SDS at either high (68° C) or low (55° C) stringency conditions. Lambda fix II genomic libraries made from human placenta (Stratagene) and Sprague Dawley rats were screened under low stringency conditions.

In situ hybridization was performed as described (Schaeren-Wiemers and Gerfin-Moser, 1993) using full-length clones VN1 to VN7 as templates to synthesize digoxigenin-labeled cRNA probes. Sequences corresponding to the BamH1-Asp718 fragment of OMP cDNA (Rogers et al., 1987) were used to synthesize a 1kb OMP probe. The sequence encompassing the transmembrane domains 3 through 7 of MOE receptor M12 was isolated by PCR.

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